Purification and Functional Properties of Simian Virus 40 Large and Small T Antigens Overproduced in Insect Cells

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The insect baculovirus *Autographa californica* nuclear polyhedrosis virus was used as an expression vector for the simian virus 40 (SV40) small t (t) and large T (T) antigens. *Spodoptera frugiperda* (SF9) cells infected with recombinant viruses encoding these proteins produced ~1 to 2 µg of t and up to 30 µg of T per 3 × 10⁶ cells. The former was highly soluble after Nonidet P-40 extraction of the infected cells, unlike its *Escherichia coli*-produced counterpart. Both SF9-produced proteins were of authentic size and could be readily immunoprecipitated by specific antibodies. Single-step immunoaffinity chromatography was used to purify the two proteins to near homogeneity, with yields averaging 70% in each case. Experiments to test the biological activity of the baculovirus SV40 proteins showed that SF9 t was capable of associating with two of the cellular proteins reported to bind to t in SV40-infected mammalian cells. Moreover, SF9 T had ATPase activity comparable to that of T produced in monkey cells, exhibited helicase activity and SV40 origin-specific DNA binding, and was active in the SV40 DNA replication assay in vitro. Thus, the SV40 T antigens produced in insect cells can be used in future studies of their biochemical roles in vitro and in vivo.

The two early-region products of simian virus 40 (SV40), large T antigen (T) and small t antigen (t), are both multifunctional proteins active in the virus-transforming process (5, 14, 15, 19, 33, 40, 48), although the mechanisms by which they function in this event are unknown. Indeed, several in vitro functional properties of each have been elucidated. Nevertheless, none has been securely linked to the role played in the transformation process by either protein, and some are believed to be unrelated (10). T antigen is required for the initiation of viral DNA replication in lytically infected cells. It is also a major regulator of viral gene expression. In vitro, the protein can bind specifically to its own replication origin (51; for a review, see reference 12), displays ATPase, helicase, and associated protein kinase activities (16, 20, 45, 50), and forms a seryl-*o*-5'-AMP adduct after incubation with ATP and Mg²⁺ (7). It is an obligate participant in the virus-transforming process.

SV40 t has a mitogenic effect in cells which also synthesize T (11, 22) and renders growing cells resistant to the cellular DNA synthesis inhibition effect of theophylline (41). Its presence correlates with the dissolution of actin cable structures in some cells (3, 18, 40), and the protein can bind specifically to a number of cell proteins in vitro. Three of them coimmunoprecipitate with t from infected monkey cell extracts (35, 53). One of these (tubulin) and two others of unknown identity (56K and 32K) can bind to crude t synthesized in mammalian cells or to t purified to homogeneity from a recombinant strain of *Escherichia coli* (4, 35). Unlike T, t is not required for SV40-induced neoplastic transformation in cultured cells or for SV40 tumor formation in virus-infected hamsters (9, 23, 26, 29). However, recent evidence implies that it can enhance the transforming activity of limiting quantities of T (1, 2). The mechanism underlying this helper effect is unknown.

In attempts to further understand the biological roles of these two proteins, various methods have been used to overproduce them in mammalian and bacterial cells. T can be overproduced by at least 5- to 10-fold at the hands of an adenovirus-SV40 recombinant virus which contains the T gene under the control of the adenovirus major late promoter (17, 49). Similarly, monkey cells infected by certain cold-sensitive mutants of SV40 overproduce T by four- to sevenfold compared with comparable wild-type virus-infected cells (e.g., cs1085; 13). These mutants contain deletions or nucleotide substitutions in sequences abutting the origin of DNA replication which play a role in the T-mediated regulation of the early transcription unit (25, 38, 47). SV40 t has been overproduced in *E. coli* by placing its coding unit under the control of a strong promoter. In the best case situation, t constituted >5% of total bacterial cell protein, but much of it was highly aggregated and insoluble, making it necessary to purify the protein in denaturing solvents (3).

Recently, a helper-independent recombinant-baculovirus expression system has been developed and adapted to the overproduction of multiple eucaryotic proteins in insect cells (27, 34, 39, 44). A key component is a mutant derivative of *Autographa californica* nuclear polyhedrosis virus (AcNPV), which, when suitably reconstructed, can lead to the high-level expression of various inserted foreign genes such as those for polyomavirus large T and the influenza virus hemagglutinin (27, 39). Recently, Jeang et al. (24) have inserted an intact SV40 early region into *A. californica*. Infection of *Spodoptera frugiperda* (SF9) cells with this virus led to the production of t but little or no T. Results of SV40 RNA analysis implied that preferential production of t messenger in these cells underlies the markedly dissimilar synthesis of these two proteins.

In this report, we describe the construction of two plasmids, pAc373t4 and pAc373T2, which contain the coding units for SV40 t and T, respectively. In both cases, the viral gene has been inserted immediately downstream of the AcNPV polyhedrin promoter. Recombinant viruses contain-

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ing these genes were isolated by using cotransfection of *SF9* with each of these plasmids and wild-type AcNPV DNA. Upon infection of *SF9* cells with these recombinant viruses, biochemically useful amounts of both t and T could be produced and purified to near homogeneity in high yield by one-step immunoaffinity chromatography (43). The purified products were found to be active in one or more functional in vitro assays.

MATERIALS AND METHODS

Cells and viruses. *S. frugiperda* cells (*SF9*) and AcNPV were obtained from Max Summers, Texas A&M University. The *SF9* cells were grown in monolayer or Spinner cultures at 27°C in Grace insect medium (GIBCO Laboratories) supplemented with 0.33% yeastolate, 0.33% lactalbumin hydrolysate (Difco Laboratories), and 10% heat-inactivated fetal calf serum (Flow Laboratories). The two recombinant baculoviruses containing the genes for SV40 T and t were isolated after cotransfection of recombinant plasmids pAc373T2 or pAc373t4 (Fig. 1) and wild-type AcNPV DNA into *SF9* cells (Method II; 46). At 4 days after transfection, supernatant medium containing virus was collected, serially diluted, and used to infect 50% confluent *SF9* cells. Infected cells were overlaid with 1.5% low-melting-point agarose (Seaplaque; FMC Bioproducts) in Grace medium. Six days after infection, plates were examined visually for the presence of nonoccluded plaques, a sign of the replication of recombinant virus. These plaques were picked, and the virus therein was used to infect fresh *SF9* cells which were subsequently lysed and immunoprecipitated with anti-T/t antibody to test for SV40 protein synthesis. Those viruses which produced t or T were then purified by four serial rounds of plaque purification.

CV-1P cells were grown in Dulbecco modified Eagle minimal essential medium containing 10% calf serum (Flow Laboratories). To produce SV40 T and t in monkey cells, the SV40 mutant cs1085 (13) was used to infect CV-1P cells at a multiplicity of infection (MOI) of 5 to 10. Infected cells were harvested 48 h postinfection for subsequent lysis and isolation of SV40 proteins.

Construction of recombinant plasmids. Standard methods of molecular cloning were used throughout (32). To generate pAc373T2, the plasmid pSP6TEX (9) was digested with *Bam*HI to yield a fragment containing a cDNA of T extending from nucleotide 5171 (a *Hind*III site converted to *Bam*HI) to nucleotide 2666 (a *Hpa*I site converted to *Bam*HI; 9). Following elution of the cDNA fragment from an agarose gel, phenol extraction, and ethanol precipitation, this DNA was ligated to pAc373 which had been linearized with *Bam*HI (Fig. 1A). Competent *E. coli* HB101 cells were transformed with the ligation mixture, and bacterial colonies were screened for the presence of recombinant plasmid by restriction enzyme analyses. In pAc373T2, the AcNPV-SV40 5' joint is located 50 base pairs downstream from the polyhedrin cap site or 8 base pairs before the polyhedrin translation initiation codon. The 3' SV40-AcNPV joint is located 177 nucleotides downstream from the polyhedrin ATG initiation codon in the polyhedrin gene. Thus, this plasmid also contains the polyhedrin coding region beginning at +177 as well as the polyhedrin poly(A) addition site. However, the SV40 T and the polyhedrin coding units are separated by the T translation stop codon and the 3' untranslated region of the T cDNA unit.

The plasmid pAc373t4 was constructed in a similar manner. The SV40 t gene was excised from the plasmid 2 *Bgl*II

FIG. 1. Construction of SV40 baculovirus vectors pAc373T2 and pAc373t4. (A) The intact SV40 LT cDNA coding unit was excised from pSP6TEX (9) by cleavage with *Bam*HI and cloned into the *Bam*HI site of pAc373 to yield pAc373T2. (B) The SV40 t coding unit was excised from plasmid 2 *Bgl*II (pMEA2*Bgl*II SV40 t; 2) by cleavage with *Bgl*II and cloned into the *Bam*HI site of pAc373 to yield pAc373t4. The resulting plasmids contained the relevant SV40 genes in the appropriate orientation with respect to the polyhedrin promoter (cf. Materials and Methods), and corresponding *E. coli* transformants were selected on ampicillin-containing agar plates. All procedures were performed as described in Materials and Methods.

t (2) by digestion with *Bgl*II and cloned into the *Bam*HI site of pAc373 as shown in Fig. 1B. The SV40 t coding sequence used in this study extends from SV40 nucleotide 5171 to 4568 and contains an intact t coding unit from which unique T coding sequences were deleted (2). The 5' and 3' AcNPV flanking sequences were identical to those present in the T plasmid construct. Thus, both SV40 genes were positioned immediately downstream from the AcNPV polyhedrin promoter in pAc373.

Radiolabeling of cells and preparation of cell extracts. Uninfected CV-1P cells or cs1085-infected CV-1P cells were rinsed once with Dulbecco modified Eagle minimal essential medium lacking methionine and serum and labeled for 4.5 h in the same medium containing 0.5% dialyzed fetal calf serum and 400 μ Ci of [35 S]methionine (800 Ci/mmol) per ml. Before extraction, cells were rinsed at 4°C with phosphate-buffered saline (PBS; 0.01 M NaPO₄ [pH 7.4], 0.14 M NaCl)

followed by wash buffer (20 mM Tris hydrochloride [pH 7.5], 0.135 M NaCl, 1 mM MgCl₂, 10% glycerol). Cells (3×10^6) were then extracted with 1 ml of lysis buffer (wash buffer plus 1% Nonidet P-40 [NP-40], 2 mM phenylmethylsulfonyl fluoride [PMSF], and 10 μ l of aprotinin per ml) per 60-mm dish for 20 min at 4°C. Insoluble debris was removed by centrifugation at 4°C. Extracts, kept on ice, were used within 1 h for the experiments outlined below.

Immunoprecipitation and polyacrylamide gel electrophoresis. Cell extracts were incubated with monoclonal antibody-containing tissue culture medium or hamster or rabbit antisera. Incubation was for 1 h at 4°C, followed by a 30-min incubation with protein A-Sepharose beads (50 μ l of a 50% suspension; Sigma Chemical Co.) at the same temperature. Beads were pelleted briefly by centrifugation and washed once with PBS, twice with 0.1 M Tris hydrochloride (pH 7.5)–0.5 M LiCl, and once with PBS. The beads were boiled in Laemmli standard sample buffer (28), and eluted proteins were analyzed by one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. For two-dimensional polyacrylamide gel electrophoresis, immunoprecipitate eluates were mixed with an equal volume of dilution buffer (16% NP-40, 2% ampholine mix [1.5% of pH 3-10 solution, 0.5% of pH 6-8 solution; LKB Instruments, Inc.], 8 M urea, 7.5% 2-mercaptoethanol), and the mixture was then saturated with solid urea (pellets; Mallinckrodt, Inc.). Gels were electrophoresed as described by O'Farrell (36, 37).

Western blotting (immunoblotting). Protein samples for blotting were electrophoresed through 10% SDS-polyacrylamide gels and transferred to nitrocellulose paper by using a Hoefer Transphor TESO apparatus. The nitrocellulose strip was blocked with 3% bovine serum albumin, 10 mM Tris hydrochloride (pH 8), 150 mM NaCl, and 0.05% Tween 20 for 1 h at 37°C. After the blot was rinsed with the same buffer lacking bovine serum albumin (TST buffer), it was incubated with PAb 419 (1:10 dilution of crude hybridoma tissue culture medium in TST buffer) overnight at room temperature. The blot was washed three times with TST buffer and incubated with a 1:7,500 dilution of anti-mouse immunoglobulin G (IgG) alkaline phosphatase conjugate (Promega) in TST buffer. Blots were assayed for color development by the suggested method of the manufacturer (Promega).

Immunoaffinity chromatography. The monoclonal antibody PAb 419 was coupled to protein A-Sepharose as described previously (43). Purification of T and t from SF9 cells was performed by a modification of the method of Simanis and Lane (43). The following buffer solutions were used: lysis buffer (50 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] [pH 9.0], 0.5% NP-40, 0.2 M sodium acetate, 1 mM dithiothreitol [DTT], 50 μ g of PMSF per ml, 10% glycerol); wash buffer I (50 mM HEPES [pH 7.8], 0.5 M sodium acetate, 1 mM DTT, 50 μ g of PMSF per ml, 10% glycerol); wash buffer II (50 mM HEPES [pH 9.0], 1 mM DTT, 50 μ g of PMSF per ml, 10% glycerol); elution buffer (20 mM triethylamine [pH 11.3], 50 μ g of PMSF per ml, 10% glycerol); and neutralizing buffer (0.5 M HEPES [pH 7.8], 10 mM DTT, 10% glycerol). Portions of fractions eluted from the column were screened for the presence and purity of T or t by SDS-polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining. Protein concentrations were determined by using an assay kit from Bio-Rad Laboratories.

ATPase assay. The ATPase assay was performed by the method of Bradley et al. (8) in a 20- μ l reaction mixture containing the buffer components used in the replication assay described below, 1.32 pmol of [γ -³²P]ATP (specific

activity, 3,000 Ci/mmol), and immunoaffinity chromatography-purified T (0.01–1.0 μ g). Reactions were incubated at 37°C for 30 min. Phosphate release (in picomoles) was measured by thin-layer chromatography of 1- μ l samples on polyethyleneimine cellulose developed in 1.2 M LiCl.

DNA binding. The SV40 origin DNA-binding assay was performed by the method of McKay (31). pSVori DNA (2,643 base pairs [52]) was digested with *Hind*III and *Cla*I and then 3' end labeled with α -thio-³⁵S-labeled dATP by using T4 polymerase. Purified T (0.25 μ g) was mixed with the labeled digest (25 ng) in 50 mM HEPES (pH 7.5), and the mixture was incubated at 4°C for 30 min. Complexes were immunoprecipitated with PAb 419 (100 μ l) and Formalin-fixed *Staphylococcus aureus* (Cowan A strain; 25 μ l of a 10% suspension in water) for 1 h. Samples were washed with PBS and eluted from the bacteria with 1% SDS. The isolated DNA was analyzed by polyacrylamide gel electrophoresis followed by autoradiography to detect the relevant fragments.

SV40 DNA replication in vitro. Assays were performed as described by Li and Kelly (30). A standard assay contained buffer components (40 mM HEPES [pH 7.8], 7 mM magnesium acetate, 0.65 mM DTT, 1 mM EGTA [ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid], 4% polyethylene glycol [14,000 molecular weight]), nucleotides (20 μ M [α -³²P]dCTP and [α -³²P]dTTP at 5 Ci/mmol; 100 μ M dATP and dGTP; 4 mM ATP; 150 μ M GTP, UTP, and CTP; plus 5 mM phosphoenolpyruvate and 3 μ g of pyruvate kinase), CV-1P cell extract (200 μ g of protein; 52), form I pSVori DNA (100 ng; 52), and immunoaffinity-purified T (0.2 to 2 μ g). Samples were incubated at 37°C for 4 h. DNA products were isolated, cleaved with *Eco*RI and *Dpn*I, and analyzed by electrophoresis in 0.8% agarose gels. Replication was expressed as picomoles of nucleotide incorporated into *Dpn*I-resistant DNA comigrating with form III pSVori DNA (30).

RESULTS

Generation of SV40 T and t recombinant baculoviruses. The scheme for constructing the T and t baculovirus vectors is shown in Fig. 1. An intact SV40 T cDNA coding unit was derived as a unique fragment from the plasmid pSP6TEX (9) by *Bam*HI digestion. It was cloned into the *Bam*HI site of pAc373 (Fig. 1A). In the ensuing plasmid, pAc373T2, this sequence was linked in the sense direction to the *A. californica* polyhedrin promoter. The SV40 t cistron and intron were isolated as a 603-base-pair unit by digestion of the plasmid 2 *Bgl*II t (pMEA2*Bgl*II SV40 t [2]; Fig. 1B) with *Bgl*II. This fragment was also cloned into the isoschizomeric *Bam*HI site of pAc373 to yield pAc373t4 (Fig. 1B). Like its T relative, pAc373t4 contains the t coding unit linked in the sense orientation to the polyhedrin promoter element. In both cases, there is an intact poly(A) addition site in the untranslated polyhedrin gene segment situated downstream of the T and t coding units (cf. Materials and Methods).

For the production of recombinant viruses, separate cultures of *S. frugiperda* cells (SF9) were cotransfected with each of the SV40-polyhedrin promoter plasmids and intact AcNPV genomic DNA. After 4 days, the medium was collected, cells were removed by centrifugation, and the residual virus was serially diluted. Plates of SF9 cells were infected with serial dilutions of the aforementioned stock, and, after 5 to 7 days, they were screened for nonoccluded plaques. These plaques were picked, and the virus contained in each was amplified in SF9 and then replaques. Three to

FIG. 2. Synthesis of SV40 T and t in recombinant baculovirus-infected *SF9* cells. Cells were either mock infected (mock) or infected (1 to 3 PFU per cell) with vAct4 (st), vAct2 (LT), or wild-type (wt) AcNPV as described in Materials and Methods. They were harvested and lysed in NP-40-containing buffer. An aliquot of each lysate (0.1%) was either electrophoresed directly through a 10% SDS-polyacrylamide gel (lanes 4, 7, 9 and 11) or immunoprecipitated with immune (PAb 419, lanes 2, 10 and 12) or an identical amount of nonimmune (anti-E1a, lane 3) mouse IgG monoclonal antibody (50 μ l of tissue culture supernatant) before SDS-polyacrylamide gel electrophoresis. Alternatively, the lysate was immunoprecipitated with a monospecific anti-T/t rabbit serum (2 μ l; lane 5) or normal rabbit serum (2 μ l; lane 6). The gel was stained with Coomassie blue. Size markers are shown in lanes 1 and 8, and their sizes are indicated on the left. The positions of t (st) and T (LT) are shown on the right. All manipulations were performed as described in Materials and Methods.

four rounds of plaque purification were required to obtain pure T (vAct2) or t (vAct4) recombinant virus free of intact AcNPV.

Synthesis of SV40 t and T in recombinant AcNPV-infected *SF9* cells. Plaque-purified vAct2 and vAct4 were used to infect *SF9* cells. Infected cells were lysed 48 to 63 h later with NP-40-containing buffer, and lysates were either directly electrophoresed through SDS-polyacrylamide gels or immunoprecipitated and then electrophoresed. Figure 2 shows a comparison of the protein profile from vAct2- and vAct4-infected and uninfected *SF9* extracts (lanes 4, 7, and 9, respectively). An extract from AcNPV-infected *SF9* cells is also shown in lane 11. Neither T nor t was visible above the background of *SF9* proteins.

When vAct2- and vAct4-infected cell extracts were immunoprecipitated with the T/t-specific monoclonal antibody PAb 419 and a monospecific anti-T/t rabbit serum, respectively, bands of 94 (Fig. 2, lane 2) and 20 (lane 5) kilodaltons (kDa) were clearly present. These proteins were absent

TABLE 1. Comparison of large T concentrations in cs1085-infected CV-1P and in vAct2-infected *SF9* cells^a

Cell line	Protein (mg/ml of cell extract)	T (μ g):	
		Per mg of crude cell protein	Per 3×10^6 cells
cs1085-CV-1P	0.63–2.50	2	3–5
vAct2-SF9			
Expt 1	0.2–0.25	7–10	3
Expt 2 ^b	1.3–1.7	18–23	~30

^a CV-1P cells were infected with cs1085 (MOI = 5 to 10), and *SF9* cells were infected with vAct2 (MOI = 1 to 3). The protein concentrations of the cell extracts were determined by the Bio-Rad protein assay. Each extract was immunoprecipitated in antibody excess with a constant volume of PAb 419 (~5 μ g), and the T concentration was estimated by comparison of Coomassie blue-stained band intensity with that of an authentic T standard.

^b Experiment 2 was carried out under improved cell culture conditions.

when the same extracts were immunoprecipitated with a suitable control antibody or serum. As additional negative controls, both uninfected and AcNPV-infected *SF9* cell extracts were immunoprecipitated with PAb 419, and no proteins of 94 or 20 kDa were present in the eluates (lanes 10 and 12). Thus, immunoreactive T and t of the expected sizes were present in the relevant baculovirus-infected cells. One can estimate from this experiment that ~1 to 2 μ g of t and 2.5 to 3 μ g of T were present in extracts of 3×10^6 infected *SF9* cells (Table 1). In more recent experiments (e.g., Table 1, experiment 2), T was produced in 10-fold-higher quantities, largely because of better control of the temperature (27°C) at which the infected cultures were incubated.

Comparison of T production in recombinant baculovirus-infected *SF9* cells and SV40-infected CV-1P cells. Since mammalian systems for overproducing SV40 T have been widely used (13, 17, 49), it was of interest to compare the amount of T protein produced in a subset of them and in vAct2-infected *SF9* cells. In particular, monkey cells infected by high multiplicities of the SV40 derivative, cs1085, are known to produce four- to sevenfold more wild-type T than a wild-type SV40-infected culture of the same type does (13). Table 1 shows the results of experiments in which the large T concentration was compared in cs1085-infected CV-1P cells and vAct2-infected *SF9* cells.

Specifically, CV-1P cells were infected with cs1085 virus (MOI 5 to 10). *SF9* cells were infected with vAct2 (MOI 1 to 3) in two different experiments. Experiment 2 (Table 1) shows later results obtained when there was better temperature control of the relevant cultures. At 48 h postinfection with cs1085 and 60 h postinfection with vAct2, 3×10^6 cells were washed with PBS and then extracted with NP-40-containing buffer. The protein content of a sample of each extract was then measured. Another sample of each extract was immunoprecipitated with an excess of PAb 419 (21). The quantity of 94-kDa T precipitated in each case was determined after SDS-polyacrylamide gel electrophoresis by relative Coomassie blue staining with authentic, intact SV40 T as a standard. The results of this analysis revealed the presence of up to 11.5-fold more SV40 T per mg of extracted protein from vAct2-infected *SF9* than from cs1085-infected CV-1P cells (Table 1). A similar analysis was performed with Ad5SVR111-infected 293 cells, which also overproduce T (17). These cells contained ~4 μ g of T per mg of cell extract protein. Thus, the concentration of T per mg of protein in vAct2-infected *SF9* cells was as much as five- to sixfold higher than that observed in Ad5SVR111-infected cells.

FIG. 3. Western blotting of extracts from vAct4-infected *SF9* cells. vAct4-infected *SF9* cells were lysed with an NP-40-containing buffer, and insoluble material was isolated by centrifugation as described in Materials and Methods. Portions (5% [by volume] of the supernatant and 5% [by volume] of the SDS-redissolved pellet fraction) from each were subjected to SDS-polyacrylamide gel electrophoresis. Following transfer of the electrophoresed proteins to nitrocellulose paper, the latter was incubated first with PAb 419 and then with alkaline phosphatase-labeled anti-mouse IgG. The nitrocellulose was then washed and incubated with a chromogenic substrate. The migration position of authentic SV40 t, as determined by gel electrophoresis of purified protein, is indicated.

Solubility of t produced in vAct4-infected *SF9* cells. Although there is an efficient *E. coli* overexpression system for SV40 t, most of the protein in the relevant bacterial extracts is insoluble. Therefore, it has been difficult to purify the protein from this source under nondenaturing conditions. To test the solubility of t synthesized in the vAct4-infected *SF9* cells, infected cells were washed, lysed in NP-40-containing buffer, and centrifuged to separate soluble protein from insoluble debris (see Materials and Methods). After the soluble fraction was separated from the pellet, the latter was washed once with lysis buffer and dissolved in boiling SDS-containing buffer. Identical quantities of the soluble and the dissolved pellet fractions from equal numbers of cells were then electrophoresed through an SDS-polyacrylamide gel, and the separated proteins were transferred to nitrocellulose for Western blotting (see Materials and Methods). Figure 3 shows the results of incubating the blotted supernatant and pellet fractions of vAct4-infected cells with PAb 419. At least 90% of the t was present in the soluble fraction. The opposite was true for a standard strain of t-overproducing *E. coli* (865i) evaluated previously (3).

Purification of SV40 T and t from infected *SF9* cells. To facilitate further studies of the biological and biochemical properties of the recombinant baculovirus-encoded SV40 proteins, we purified each by a modification of the immunoaffinity chromatography method of Simanis and Lane (43; cf. Materials and Methods). vAct2- and vAct4-infected *SF9* cells were extracted in NP-40-containing buffer, and the

FIG. 4. Immunoaffinity purification of SV40 t and T from infected *SF9* cells. The total soluble NP-40 extracts (2 ml) from $\sim 4 \times 10^7$ vAct2- and vAct4-infected *SF9* cells were individually prepared and preincubated with protein A-linked Sepharose 4B to remove proteins binding nonspecifically to Sepharose. t (A) and T (B) were then purified by immunoaffinity chromatography on protein A-Sepharose 4B coupled to PAb 419 as described in Materials and Methods. The immunoabsorbent was washed extensively, and the bound t or T was eluted with 20 mM TEA (pH 11.3) as described by Simanis and Lane (43). (A) Column fractions of the TEA eluate of a column loaded with a vAct4-infected *SF9* extract. One-fourth of each fraction was electrophoresed through a 10% SDS-polyacrylamide gel which was subsequently fixed and stained with Coomassie blue. The gel shows column fractions 1 to 12 (lanes 1 to 12, respectively), one-fourth of the boiling 2% SDS eluate of the column after prior TEA elution (lane 13), and an aliquot (0.005%) of the crude extract which was originally loaded onto the column (lane 15). The migration position of homogeneous t from *E. coli* 865i (3) is shown in lane 14. (B) T synthesized in vAct2-infected *SF9* cells, purified by the immunoaffinity method, electrophoresed and stained with Coomassie blue, is shown in lane 17. Purification steps were performed as described in Materials and Methods. Molecular weight markers are shown in lane 16.

soluble fraction of each was preabsorbed with protein A-Sepharose CL-4B to remove proteins binding nonspecifically to the Sepharose. Each preabsorbed extract was applied to a separate column of PAb 419 linked to protein A-Sepharose. After the unbound protein was washed away, the bound protein was eluted with 20 mM triethylamine (TEA), pH 11.3. Samples from the TEA eluate of the column loaded with vAct4-infected *SF9* extract are shown in Fig. 4A. One-fourth of each column fraction was electrophoresed through an SDS-polyacrylamide gel which was then stained with Coomassie blue. Small t eluted from the column in fractions 6 to 11 (lanes 6 to 11). Lane 13 shows the spectrum of proteins remaining on the TEA-treated column after boiling 25% of it in SDS-containing buffer, and lane 15 shows an aliquot (0.005%) of the crude extract which was originally loaded onto the column. The results indicate that the column effectively bound all the SV40 t in the vAct4-infected *SF9* crude extract, since the column flowthrough contained little or no material which reacted with PAb 419 (data not shown). Furthermore, apparently homogeneous, immunoreactive

FIG. 5. Coprecipitation of cellular 56K and 32K proteins with *SF9*-derived SV40 t. (A) An NP-40 extract (~25 μ g) of vAct4-infected *SF9* cells was incubated with an 35 S-labeled extract of confluent CV-1P cells (0.5 ml; 50% of the extract of a 100-mm dish) for 30 min at room temperature. The mixture was then immunoprecipitated with 2 μ l of hamster anti-SV40 tumor serum (provided by K. Rundell) as described in Materials and Methods. (B) An NP-40 extract (~25 μ g) of vAct2-infected *SF9* cells was incubated with an 35 S-labeled extract of CV-1P cells (0.5 ml) and immunoprecipitated with antitumor serum. (C and D) NP-40 extracts of 35 S-labeled cs1085-infected CV-1P cells were incubated with either hamster antitumor serum (3 μ l; panel C) or normal hamster serum (3 μ l; panel D). All immunoprecipitates were analyzed by two-dimensional gel electrophoresis. The migration positions of the 56K and 32K cell proteins are marked with arrows. A, Actin; T, tubulin.

20K protein, which comigrated with authentic t, was eluted with TEA. Analogous results were obtained for T in the purification experiment performed with vAct2-infected *SF9* cell extracts (Fig. 4B). The results further showed that ~60 to 70% of the t or T in the relevant *SF9* extracts could be recovered as a purified product by this approach (data not shown).

Biological activity of t from infected *SF9* cells. One criterion of whether t is present in its native configuration has been its ability or inability to bind specifically to selected cellular proteins. In particular, undenatured, monomeric SV40 t, produced in *E. coli*, can bind specifically to two proteins of 56 and 32 kDa present in crude extracts of CV-1P cells (4). Moreover, native SV40 t as well as other nondenatured papovavirus small t species synthesized in animal cells are believed to form stable complexes with these proteins (4, 53). The results suggested that 56K and 32K binding is a functional property of nondenatured t. Given these observations, we asked whether t synthesized in *SF9* cells could bind these two proteins. Specifically, unlabeled vAct4-infected *SF9* cells were extracted, and the extracts were incubated with an [35 S]methionine-labeled extract of CV-1P cells for 30 min at room temperature. This mixture was then immunoprecipitated with hamster antitumor serum (generously provided by K. Rundell). As a negative control, an extract from T-containing *SF9* cells was treated identically in parallel. As positive controls, extracts of cs1085-infected CV-1P cells were simultaneously immunoprecipitated with antitumor serum (Fig. 5C) and with normal hamster serum (Fig. 5D). Immunoprecipitated proteins were electrophoresed through two-dimensional gels (36), and the dried gels were autoradiographed. Comparison of panels C and D from Fig. 5 shows the appearance of the aforementioned 56K and 32K t-binding proteins (indicated by arrows) in the cs1085-

infected cell extract. Neither t nor T focused in the first dimension and, hence, do not appear in these autoradiograms. A comparison of panels A and C shows that the aforementioned cellular proteins were specifically immunoprecipitated from a preincubated mixture of extracts of t-producing *SF9* and uninfected CV-1P cells. By contrast, neither protein was immunoprecipitated from a mixture of the same uninfected CV-1P extract and the T-containing *SF9* extract (panel B). The difference in the ratio of the 56K and 32K proteins between panels A and C seems to be an artifact of the gel system and may not be meaningful (D. Pallas and C. Murphy, unpublished observations). Nevertheless, these experiments show that t produced in recombinant baculovirus-infected cells can specifically complex with two known t-binding proteins.

Biological activity of T from infected *SF9* cells. T purified to near homogeneity from infected *SF9* cells was tested for three different, intrinsic biochemical activities: specific DNA binding, ATPase, and SV40 DNA replication in vitro. To measure origin-specific DNA binding activity, a modification of the method of McKay (31) was performed. As shown in Fig. 6, the purified protein demonstrated clear and specific binding of the relevant, origin-containing restriction fragment among the mixture of fragments tested. The ATPase activities of T purified by immunoaffinity chromatography from vAct2-infected *SF9* cells and from cs1085-infected CV-1P cells were assayed in parallel. The results depicted in Fig. 7 show that both proteins displayed comparable ATPase activity over the range of protein concentrations tested.

In the in vitro DNA replication assays (cf. Materials and Methods), DNA products were isolated, cleaved with *Eco*RI and *Dpn*I to digest anything other than newly synthesized DNA into small fragments, and then analyzed by electrophoresis in a 0.8% agarose gel. Replication of input DNA

FIG. 6. Origin-specific DNA binding of insect cell-derived SV40 T. T (250 ng) purified from vAct2-infected *SF9* cells (lane 1) and from cs1085-infected CV-1P cells (lane 2) was incubated with a ^{35}S -labeled *HindIII*-*ClaI* digest of pSVori DNA and immunoprecipitated as described in Materials and Methods. Lane 3 is an electropherogram of the labeled DNA digest used in these analyses and run in the same gel as the immunoprecipitated samples. The arrow marks the origin-containing fragment.

(Fig. 8) was reflected by the appearance of a radioactive band of DNA migrating to the same position as pSVori form III DNA (marked by an arrow). Figure 8 shows a comparison of the replication activities of T purified from *SF9* (lanes 2 to 6) and CV-1P (lanes 7 to 10) cells. The quantity of T added to each replication reaction is noted above each lane. As measured by this assay, the replication activity of T from vAct2-infected *SF9* cells was similar to that purified from cs1085-infected cells. The difference in replication seen at higher concentrations of T is due to inhibitory contaminants copurifying with the fraction of T used in this experiment. Other purified preparations of T from extracts of vAct2-infected *SF9* cells have shown no inhibition when $\geq 1.5 \mu\text{g}$ of T is used (data not shown). In keeping with the finding that purified T from *SF9* is replication competent, we have also

FIG. 7. ATPase activity of purified T synthesized in *SF9* and in CV-1P cells. ATPase assays were performed by using increasing amounts of each immunoaffinity-purified T species (noted along the abscissa). Samples were analyzed by thin-layer chromatography after 5, 10, 15, 30, and 60 min of incubation at 37°C . The quantity of P_i released by each T species is shown after a 30-min incubation. These two proteins also demonstrated similar specific ATPase activities at the other times indicated above (data not shown).

FIG. 8. Comparison of the in vitro DNA replication activities of purified T synthesized in *SF9* and in CV-1P cells. DNA products were isolated from standard replication reactions containing purified vAct2-T (lanes 2 to 6) and SV40-cs1085 T (lanes 7 to 10). The reaction products were generated and electrophoresed as described in Materials and Methods and identified by autoradiography. Nucleotide incorporated into newly synthesized DNA was calculated from the radioactivity present in the *DpnI*-resistant band, which comigrated with form III pSVori DNA (30). Amount incorporated by lanes: 1 (no T present), 0 pmol; 2 to 6, 9, 82, 42, 27, and 26 pmol, respectively; 7 to 10, 5, 72, 84, and 46 pmol, respectively. The amount of T present in each reaction mixture is shown across the top of the figure. The arrow indicates the migration position of form III pSVori DNA.

detected helicase activity in a purified preparation of baculovirus-encoded T (data not shown). Thus, in multiple assays of function, vAct2 T behaved similarly to authentic T synthesized in monkey cells.

DISCUSSION

A recombinant *A. californica* vector has been used to overproduce the two SV40 early region gene products, T and t. Both could be generated as apparently intact molecules and in potentially useful biochemical amounts. Moreover, each protein could be purified to homogeneity by an established, single-step immunoaffinity chromatography procedure developed earlier by others (43). Yields of each purified protein were high ($\sim 70\%$) where quantitated, and the time required to isolate these proteins from the end of the period of cell culture to the completion of purification was short (~ 6 h), thereby minimizing the risk of losses arising from long periods of manipulation. The yields of T and t were considerably higher than those observed in the case of either of two mammalian cell production systems (13, 49). Indeed, Coomassie blue-stained bands of t were not observed in the immunoaffinity-purified products of either cs1085-infected CV-1P cells or Ad5SVR111-infected 293 cells, while they were easily detected when comparable numbers of vAct4-infected *SF9* cells were analyzed. The relatively high yield of T per milligram of *SF9*-derived protein is a significant advantage in the purification of T from baculovirus-infected cells.

T isolated from *SF9* was indistinguishable from that synthesized in CV-1P cells with respect to size, immunoreactiv-

ity, and its behavior in multiple assays of function. In addition, the fact that, where tested, its specific activity in the relevant assays was comparable to that of native T from permissive cells implies that it has retained significant elements of native secondary, tertiary, and possibly quaternary structure. The suggestion that it may well be characterized by some degree of normal oligomeric structure comes from the observation that it was as active as monkey cell-derived T in ATPase and replication assays. Both of these activities have been shown to require the ability to form specific oligomeric species of the protein (6, 42). Therefore, preliminary analyses of the oligomeric properties of T purified from vAct2-infected SF9 cells were performed by using zonal sedimentation through glycerol gradients as an assay for molecular size. In these experiments, it appeared that the distribution of oligomeric species was similar to that of T purified from cs1085-infected CV-1P cells analyzed in parallel (B. Weiner and M. Bradley, data not shown).

Small t could be purified to homogeneity under nondenaturing conditions. In the past, relatively high yields of this protein could be obtained by dissolving a pellet of t, isolated following lysis in non-denaturing buffer of an overproducing clone of *E. coli*, in SDS- or guanidine-containing buffer. Following electrophoresis or chromatography of this material in the same denaturing solution and then elimination of the denaturant from the final product, homogeneous t with some measureable biological activity could be prepared. Unlike *E. coli* t, the baculovirus-encoded protein was largely soluble in the initial crude extract, eliminating the need to enhance solubility (and hence yield) by disrupting insoluble aggregates in denaturing solvents. As an indication that crude SF9-synthesized t has at least some native structure, it was possible to show that it could form stable complexes in vitro with two cell proteins known to interact specifically with nondenatured t in mammalian cells. Interestingly, these same two proteins can be shown to interact with both polyomavirus t and middle T (D. Pallas and T. Roberts, personal communication). Thus, these three papovaviral proteins may share a common function or be affected by one or more cell processes in a common way.

Given the relative ease with which SF9 cells and recombinant AcNPV can be cultivated, and the high yields of the two T/t antigens which can be obtained from them, the baculovirus expression system may prove a useful approach to the preparation of these proteins for future biochemical studies. Nevertheless, more analytical experiments will be needed to learn how similar or different they may be to the comparable mammalian cell products in expressing those functions which, although not understood biochemically, underlie their role in the transforming process.

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